



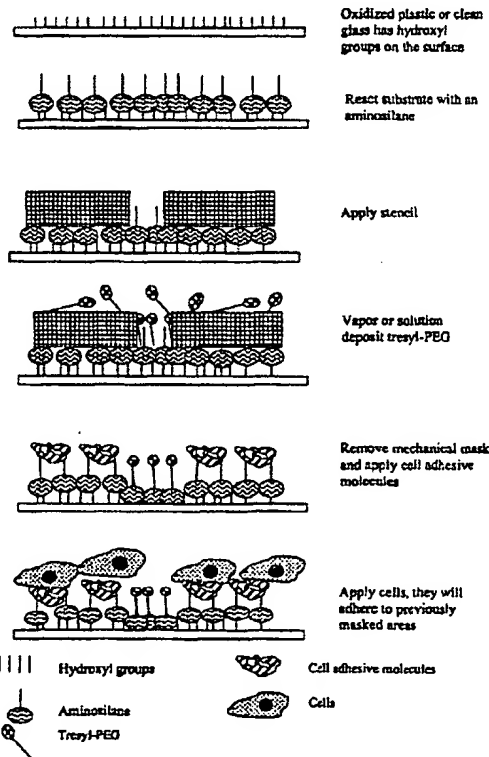
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 7 :</b> <b>G01N 33/543</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/60356</b> <b>(43) International Publication Date:</b> 12 October 2000 (12.10.00)
<b>(21) International Application Number:</b> PCT/US00/08767 <b>(22) International Filing Date:</b> 31 March 2000 (31.03.00) <b>(30) Priority Data:</b> 60/127,339 1 April 1999 (01.04.99) US 60/138,119 7 June 1999 (07.06.99) US <b>(71) Applicant (for all designated States except US):</b> CELLOMICS, INC. [US/US]; 635 William Pitt Way, Pittsburgh, PA 15238 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KAPUR, Ravi [IN/TN]; 2942 E. Bardoneer Road, Gibsonsia, PA 15044 (US). ADAMS, Terri [US/US]; 100 Eastern Avenue, Pittsburgh, PA 15215 (US). <b>(74) Agent:</b> HARPER, David, S.; McDonnell, Boehnen, Hulbert & Berghoff, Suite 3200, 300 South Wacker Drive, Chicago, IL 60606 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>

(54) Title: MINIATURIZED CELL ARRAY METHODS AND APPARATUS FOR CELL-BASED SCREENING

## (57) Abstract

The present invention describes novel methods for making a substrate for selective cell patterning, and the substrates themselves, wherein the method comprises contacting reactive hydroxyl groups on the surface of a substrate with a hydroxyl-reactive bifunctional molecule to form a monolayer, and using stencils to deposit cell repulsive or cell adhesive moieties in controlled locations on the cell culture substrate. Methods comprising selective differentiation of stem cells to create tissue specific and organ-specific cell substrates, as well as the cell substrates themselves are also provided.



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## **Miniaturized Cell Array Methods and Apparatus for Cell-Based Screening**

### **Related Applications**

This application claims priority to U.S. Provisional Patent Application Serial Nos. 60/127,339 filed April 1, 1999 and 60/138,119 filed June 7, 1999; is a continuation-in-part of U.S. Patent Application S/N 09/401,212, filed September 22, 1999, which is a continuation in part of U.S. Patent Application S/N 08/865,341 filed May 29, 1997; and is related to U.S. Patent Application Serial Nos. 09/468,673 filed 12/21/99.

### **Field of the Invention**

The present invention relates to methods and devices for cell-based high throughput and high biological content screening.

### **Background of the Invention**

In the expanding arena of drug discovery and combinatorial chemistry to generate candidate compounds, it would be very useful to be able to rapidly screen a large number of substances, via a high throughput screen, for their physiological impact on animals and humans. Before testing the efficacy of a "partially qualified" drug candidate on animals, the drug could first be screened for its biological activity and potential toxicity with living cells. The physiological response to the drug candidate could then be anticipated from the results of these cell screens.

Traditionally, "lead compounds" have moved quickly to extensive animal studies that are both time-consuming and costly. Furthermore, extensive drug testing in animals is becoming less culturally acceptable. Screening drug candidates according to their interaction with living cells, prior to animal studies, can reduce the number of animals required in subsequent drug screening processes by eliminating some drug candidates before going to animal trials. However, manipulation and analysis of drug-cell interactions using current methods does not allow for both high throughput and high biological content screening, due to the small number of cells and compounds that can be analyzed in a given period of time, the cumbersome methods required for compound delivery, and the large volumes of compounds required for testing.

High throughput screening of nucleic acids and polypeptides has been achieved using DNA chip technologies. In typical DNA analysis methods, DNA sequences of 10 to 14 nucleotides are attached in defined locations (or spots), up to tens of thousands in number, on a small glass plate. (U.S. Patent No. 5,556,752, hereby incorporated by reference). This  
5 creates an array of spots of DNA on a given glass plate. The location of a spot in the array provides an address for later reference to each spot of DNA. The DNA sequences are then hybridized with complementary DNA sequences labeled with fluorescent molecules. Signals from each address on the array are detected when the fluorescent molecules attached to the hybridizing nucleic acid sequences fluoresce in the presence of light. These devices have  
10 been used to provide high throughput screening of DNA sequences in drug discovery efforts and in the human genome sequencing project. Similarly, protein sequences of varying amino acid lengths have been attached in discrete spots as an array on a glass plate. (U.S. Patent 5,143,854, incorporated by reference herein).

The information provided by an array of either nucleic acids or amino acids bound to  
15 glass plates is limited according to their underlying "languages". For example, DNA sequences have a language of only four nucleic acids and proteins have a language of about 20 amino acids. In contrast, a living cell, which comprises a complex organization of biological components, has a vast "language" with a concomitant multitude of potential interactions with a variety of substances, such as DNA, RNA, cell surface proteins,  
20 intracellular proteins and the like. Because a typical target for drug action is with and within the cells of the body, cells themselves provide an extremely useful screening tool in drug discovery when combined with sensitive detection reagents. It thus would be most useful to have high throughput, high content screening devices to provide high content spatial information at the cellular and subcellular level as well as temporal information about  
25 changes in physiological, biochemical and molecular activities.

### Microarrays of cells

Methods have been described for making micro-arrays of a single cell type on a common substrate for other applications. One example of such methods is photochemical  
30 resist-photolithography (Mrksich and Whitesides, Ann. Rev. Biophys. Biomol. Struct. 25:55-78, 1996), in which a glass plate is uniformly coated with a photoresist and a photo mask is placed over the photoresist coating to define the "array" or pattern desired. Upon exposure to

light, the photoresist in the unmasked areas is removed. The entire photolithographically defined surface is uniformly coated with a hydrophobic substance, such as an organosilane, that binds both to the areas of exposed glass and the areas covered with the photoresist. The photoresist is then stripped from the glass surface, exposing an array of spots of exposed  
5 glass. The glass plate then is washed with an organosilane having terminal hydrophilic groups or chemically reactable groups such as amino groups. The hydrophilic organosilane binds to the spots of exposed glass with the resulting glass plate having an array of hydrophilic or reactable spots (located in the areas of the original photoresist) across a hydrophobic surface. The array of spots of hydrophilic groups provides a substrate for non-  
10 specific and non-covalent binding of certain cells, including those of neuronal origin (Kleinfeld et al., J. Neurosci. 8:4098-4120, 1988).

In another method based on specific yet non-covalent interactions, stamping is used to produce a gold surface coated with protein adsorptive alkanethiol. (U.S. Patent No. 5,776,748; Singhvi et al., Science 264:696-698, 1994). The bare gold surface is then coated  
15 with polyethylene-glycol-terminated alkanethiols that resist protein adsorption. After exposure of the entire surface to laminin, a cell-binding protein found in the extracellular matrix, living hepatocytes attach uniformly to, and grow upon, the laminin coated islands (Singhvi et al. 1994). An elaboration involving strong, but non-covalent, metal chelation has been used to coat gold surfaces with patterns of specific proteins (Sigal et al., Anal. Chem.  
20 68:490-497, 1996). In this case, the gold surface is patterned with alkanethiols terminated with nitriloacetic acid. Bare regions of gold are coated with tri(ethyleneglycol) to reduce protein adsorption. After adding  $\text{Ni}^{2+}$ , the specific adsorption of five histidine-tagged proteins is found to be kinetically stable.

More specific single cell-type binding can be achieved by chemically crosslinking  
25 specific molecules, such as proteins, to reactable sites on the patterned substrate. (Aplin and Hughes, Analyt. Biochem. 113:144-148, 1981). Another elaboration of substrate patterning optically creates an array of reactable spots. A glass plate is washed with an organosilane that chemisorbs to the glass to coat the glass. The organosilane coating is irradiated by deep UV light through an optical mask that defines a pattern of an array. The irradiation cleaves  
30 the Si-C bond to form a reactive Si radical. Reaction with water causes the Si radicals to form polar silanol groups. The polar silanol groups constitute spots on the array and are further modified to couple other reactable molecules to the spots, as disclosed in U.S. Patent

No. 5,324,591, incorporated by reference herein. For example, a silane containing a biologically functional group such as a free amino moiety can be reacted with the silanol groups. The free amino groups can then be used as sites of covalent attachment for biomolecules such as proteins, nucleic acids, carbohydrates, and lipids. The non-patterned covalent attachment of a lectin, known to interact with the surface of cells, to a glass substrate through reactive amino groups has been demonstrated (Aplin & Hughes, 1981). The optical method of forming a micro-array of a single cell type on a support requires fewer steps and is faster than the photoresist method, (i.e., only two steps), but it requires the use of high intensity ultraviolet light from an expensive light source.

In all of these methods, the result is a micro-array of a single cell type, since the biochemically specific molecules are bound to the micro-patterned chemical array uniformly. In the photoresist method, cells bind to the array of hydrophilic spots and/or specific molecules attached to the spots which, in turn, bind cells. Thus cells bind to all spots in the array in the same manner. In the optical method, cells bind to the array of spots of free amino groups by adhesion. There is little or no differentiation between the spots of free amino groups. Again, cells adhere to all spots in the same manner, and thus only a single type of cell interaction can be studied with these cell arrays because each spot on the array is essentially the same as another. Such cell arrays are inflexible in their utility as tools for studying a specific variety of cells in a single sample or a specific variety of cell interactions. Thus, there exists a need for arrays of multiple cell types on a common substrate, in order to increase the number of cell types and specific cell interactions that can be analyzed simultaneously, as well as methods of producing these micro-arrays of multiple cell types on a common substrate, in order to provide for high throughput and high biological content screening of cells.

#### **Optical reading of cell physiology**

Performing a high throughput screen on many thousands of compounds requires parallel handling and processing of many compounds and assay component reagents. Standard high throughput screens use homogeneous mixtures of compounds and biological reagents along with some indicator compound, loaded into arrays of wells in standard microplates with 96 or 384 wells. (Kahl et al., J. Biomol. Scr. 2:33-40, 1997). The signal measured from each well, either fluorescence emission, optical density, or radioactivity,

integrates the signal from all the material in the well giving an overall population average of all the molecules in the well. This type of assay is commonly referred to as a homogeneous assay.

U.S. Patent No. 5,581,487 describes an imaging plate reader that uses a CCD detector (charge-coupled optical detector) to image the whole area of a 96 well plate. The image is analyzed to calculate the total fluorescence per well for homogeneous assays.

Schroeder and Neagle describe a system that uses low angle laser scanning illumination and a mask to selectively excite fluorescence within approximately 200 microns of the bottoms of the wells in standard 96 well plates in order to reduce background when imaging cell monolayers. (J. Biomol. Scr. 1:75-80, 1996). This system uses a CCD camera to image the whole area of the plate bottom. Although this system measures signals originating from a cell monolayer at the bottom of the well, the signal measured is averaged over the area of the well and is therefore still considered a homogeneous measurement, since it is an average response of a population of cells. The image is analyzed to calculate the total fluorescence per well for cell-based homogeneous assays.

Proffitt et. al. (Cytometry 24:204-213, 1996) describe a semi-automated fluorescence digital imaging system for quantifying relative cell numbers *in situ*, where the cells have been pretreated with fluorescein diacetate (FDA). The system utilizes a variety of tissue culture plate formats, particularly 96-well microplates. The system consists of an epifluorescence inverted microscope with a motorized stage, video camera, image intensifier, and a microcomputer with a PC-Vision digitizer. Turbo Pascal software controls the stage and scans the plate taking multiple images per well. The software calculates total fluorescence per well, provides for daily calibration, and configures for a variety of tissue culture plate formats. Thresholding of digital images and the use of reagents that fluoresce only when taken up by living cells are used to reduce background fluorescence without removing excess fluorescent reagent..

A variety of methods have been developed to image fluorescent cells with a microscope and extract information about the spatial distribution and temporal changes occurring in these cells. A recent article describes many of these methods and their applications (Taylor et al., Am. Scientist 80:322-335, 1992). These methods have been designed and optimized for the preparation of small numbers of specimens for high spatial

and temporal resolution imaging measurements of distribution, amount and biochemical environment of the fluorescent reporter molecules in the cells.

Treating cells with dyes and fluorescent reagents, imaging the cells, and engineering the cells to produce a fluorescent reporter molecule, such as modified green fluorescent protein (GFP), are useful detection methods (Wang et al., In Methods in Cell Biology, New York, Alan R. Liss, 29:1-12, 1989). The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has an excitation maximum at 395 nm, an emission maximum at 510 nm and does not require an exogenous factor. Uses of GFP for the study of gene expression and protein localization are discussed in Chalfie et al., Science 263:802-805, 1994. Some properties of wild-type GFP are disclosed by Morise et al. (Biochemistry 13:2656-2662, 1974), and Ward et al. (Photochem. Photobiol. 31:611-615, 1980). An article by Rizzuto et al. (Nature 358:325-327, 1992) discusses the use of wild-type GFP as a tool for visualizing subcellular organelles in cells. Kaether and Gerdes (FEBS Letters 369:267-271, 1995) report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng (FEBS Letters 369:331-334, 1995), while GFP expression in Drosophila embryos is described by Davis et al. (Dev. Biology 170:726-729, 1995). U. S. Patent No. 5,491,084, incorporated by reference herein, discloses expression of GFP from *Aequorea victoria* in cells as a reporter molecule fused to another protein of interest. Mutants of GFP have been prepared and used in several biological systems. (Hasselhoff et al., Proc. Natl. Acad. Sci. 94:2122-2127, 1997; Brejc et al., Proc. Natl. Acad. Sci. 94:2306-2311, 1997; Cheng et al., Nature Biotech. 14:606-609, 1996; Heim and Tsien, Curr. Biol. 6:178-192, 1996; Ehrig et al., FEBS Letters 367:163-166, 1995).

The ARRAYSCAN™ System, as developed by Cellomics, Inc. (U.S. Patent No. 5,989,835) and U.S. Application Serial No. 09/031,271 filed February 27, 1998; both incorporated by reference herein in their entirety) is an optical system for determining the distribution, environment, or activity of luminescently labeled reporter molecules on or in cells for the purpose of screening large numbers of compounds for specific biological activity. The ARRAYSCAN™ System involves providing cells containing luminescent reporter molecules in an array of locations and scanning numerous cells in each location, converting the optical information into digital data, and utilizing the digital data to determine the distribution, environment or activity of the luminescently labeled reporter molecules in



the cells. The ARRAYSCAN™ System includes apparatus and computerized method for processing, displaying and storing the data, thus augmenting drug discovery by providing high content cell-based screening, as well as combined high throughput and high content cell-based screening, in a large microplate format.

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### Microfluidics

Efficient delivery of solutions to an array of cells attached to a solid substrate is facilitated by a microfluidic system. Methods and apparatus have been described for the precise handling of small liquid samples for ink delivery (U.S. Patent No. 5,233,369; U.S. Patent No. 5,486,855; U.S. Patent No. 5,502,467), biosample aspiration (U.S. Patent No. 4,982,739), reagent storage and delivery (U.S. Patent No. 5,031,797), and partitioned microelectronic and fluidic device array for clinical diagnostics and chemical synthesis (U.S. Patent No. 5,585,069). In addition, methods and apparatus have been described for the formation of microchannels in solid substrates that can be used to direct small liquid samples along the surface (U.S. Patent No. 5,571,410; U.S. Patent No. 5,500,071; U.S. Patent No. 4,344,816,).

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For purposes of integrated high throughput and high content cell based screening, particularly for live-cell imaging, an optimal microfluidic device would comprise a fluidic architecture that permits the closest possible well spacing (i.e.: highest possible well density), wherein the fluidic architecture is integrated with the cell array substrate to permit efficient fluid delivery to the cells, and eliminating the need for pipetting fluids in and out of wells. Such optimal microfluidic devices would be advantageous for cell arrays with sub-millimeter inter-well distances because it is unwieldy, if not impossible, to pipette fluids with such a high degree of spatial resolution and accuracy. Furthermore, such integrated devices could be directly used for cell based screening, without the need to remove the cell substrate from the fluidic architecture for imaging the cells.

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An optimal microfluidic device for cell based screening might further comprise a closed chamber to permit environmental control of the cells, and preferably would not directly expose the cells to electro-kinetic forces, which may affect the physiology of the cells on the substrate. For example, electrohydrodynamic pumping is less effective with polar solvents (Marc Madou, Fundamentals of Microfabrication, CRC Press, Boca Raton,

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1997, p. 433). Electro-osmosis is typically accompanied by some degree of electrophoretic separation of charged medium components, such as proteins.

U.S. Patent No. 5,603,351 ('the 351 patent') describes a microfluidic device that uses a multilevel design consisting of two upper levels with channels and a bottom level with reaction wells. However, this device is not designed for use in cell based screening. The '351 patent does not disclose a substrate containing cells or cell binding sites. The disclosed microfluidic network is designed to allow two or more reagents to be combined in a reaction well, as opposed to an optimal cell screening microfluidic system that allows living cells cultured on the well bottoms to be exposed in serial fashion to two or more different fluids. The '351 patent discloses a device with the wells etched into the substrate at a maximal well density of 50 wells/inch<sup>2</sup>. Furthermore, the substrate must be detached from the fluidic array for incubation and/or analysis. Finally, the '351 patent discloses a system of electrically-controlled electrohydrodynamic valves within the matrix of the wells that are less effective with aqueous media used in cell culture, and also may limit the degree of close-spacing between wells in the array of wells.

U.S. Patent No. 5,655,560 discloses a clog-free valving system, comprising a fluid distribution system with multiple inputs and multiple outputs incorporating a crossed array of microchannels connected vertically at crossing points by teflon valves. However, this patent does not disclose a substrate containing a cell array, nor an integrated fluidic device in combination with the substrate, nor a well density that is optimal for cell-based screening.

U.S. Patent 5,900,130 (the '130' patent) describes the active, electronic control of fluid movement in an interconnected capillary structure. This patent does not teach a fluidic architecture that maximizes the area of the cell substrate that can be occupied by cell binding sites. Nor does this patent disclose a substrate containing a cell array, nor an integrated fluidic device in combination with the substrate. Furthermore, the patent only teaches the control of fluid flow by application of an electric field to the device.

U.S. Patent 5,910,287 describes multi-well plastic plates for fluorescence measurements of biological and biochemical samples, including cells, limited to plates with greater than 864 wells. This patent does not describe a microfluidic device with a fluidic architecture integrated with the cell array substrate. Nor does the patent disclose a closed chamber to permit environmental control of the cells on the substrate.

Thus, none of these prior microfluidic devices provide a fluidic architecture that permits the closest possible well spacing (i.e.: highest possible well density), wherein the fluidic architecture is integrated with the cell array substrate to permit efficient fluid delivery to the cells, and thus eliminating the need for pipetting fluids in and out of wells.

5 Furthermore, prior microfluidic devices that comprise an array of wells use electrically-controlled electrohydrodynamic valves within the matrix of the wells that would be less effective if used with aqueous media for cell culture, and which limit the well density.

While the above advances in cell array, optical cell physiology reading, and microfluidic technologies provide supporting technologies that can be applied to improved

10 high throughput and high content cell-based screening, there remains a need in the art for integrated devices and methods that further decrease the amount of time necessary for such screening, as well as for devices and methods that further improve the ability to conduct high throughput and high content cell-based screening and the ability to flexibly and rapidly switch from one to the other. In particular, devices and methods that maximize the well

15 density, thereby increasing the number of wells that can be imaged in at one time, and thus greatly increasing the throughput of a screen while maintaining adequate resolution of the image, would be very advantageous.

The drug discovery industry already uses 96- and 384-well microplates and is in transition towards the use of 1536-well plates. However, further increases in well density

20 using prior technology are unlikely because of the great difficulty of pipetting liquids in and out of very small diameter wells.

### Summary of the Invention

The present invention fulfills the need in the art for devices and methods that

25 decrease the amount of time necessary to conduct cell-based screening, and specifically combines the ability to conduct high throughput and high content cell-based screening and to flexibly and rapidly switch from one to the other. The invention provides devices and methods for maximizing the number of wells that can be imaged at one time while still obtaining adequate pixel resolution in the image. This result has been achieved through the

30 use of fluidic architectures that maximizes well density. The present invention thus provides a miniaturized microplate system with closed fluidic volumes that are internally supplied with

Patent No. 5,077,085), thiols (U.S. Patent No. 5,776,748), and azidos (U.S. Patent No. 5,593,814). These methods provide selective localization of cells using a multi-step, equipment intensive process, and/or irreproducible techniques such as deep ultraviolet ablation of molecules, and/or printing by mechanical stamping, and/or require a polymer layer tens of nanometers (nm) in thickness, which changes the optical quality of the substrate.

Thus, there is a need in the art for affordable, facile, equipment insensitive, reproducible methods for cell patterning on durable substrates such as glass and plastic that do not decrease the optical quality of the substrate, as well as for the cell patterning substrates themselves. Most surface modification processes for glass and silicon wafers are not amenable to plastic due to the nature of the harsh solvents used. Thiols are unsuitable for coating on plastics as they require a coinage metal for forming a coordination bond with the substrate. Silanes, though amenable to coating on plastics, require a hydroxylated surface, such as presented by glass and silicon, to form a covalent bond with the substrate.

Thus, in one aspect, the present invention provides novel methods for making a substrate for selective cell patterning. In one embodiment, the method comprises

- a) providing a substrate with a surface, wherein the surface contains reactive hydroxyl groups;
- b) contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to form a monolayer;
- c) applying a stencil to the substrate;
- d) applying an electrophilic cell repulsive moiety to exposed regions of the monolayer to form a covalent bond between the cell repulsive moiety and the bifunctional nucleophile deposited in step b to form cell repulsive locations;
- e) removing the stencil; and
- f) applying cell adhesive molecules to the substrate to produce cell binding locations, wherein the cell adhesion molecules bind the substrate only in positions that were contacted by the stencil.

In another embodiment, the method comprises

- a) providing a substrate with a surface, wherein the surface comprises reactive hydroxyl groups on the surface of the substrate;

- b) contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to form a monolayer
- c) applying a stencil to the substrate;
- 5 d) applying cell adhesive molecules to exposed regions on the monolayer to produce cell binding locations;
- e) removing the stencil; and
- f) applying an electrophilic cell repulsive moiety to the substrate to produce cell repulsive locations, wherein the cell repulsive moiety forms a covalent bond with the
- 10 bifunctional nucleophile deposited in step b only in positions that were contacted by the stencil.

The surface reactive hydroxyl groups can either be naturally occurring, or can be introduced via any technique known in the art. For example, polymer or glass can be

15 oxidized so that they present surface hydroxyl groups, which react with organosilanes to produce covalent Si-O-substrate (siloxane) linkages. (U.S. Patent No. 5,077,085)

In a preferred embodiment, oxidation is accomplished by oxygen plasma treatment, which can be achieved using oxygen doped radio frequency glow discharge. This discharge is accomplished with an instrument that can produce charged particles (electrons and positive

20 ions) that interact with the background gas (oxygen), to produce free radicals under the time-varying electric field in radio frequency. The sample is placed into a cylindrical reactor, a minimal amount of oxygen gas is introduced, and charged particles are evolved between parallel-plated electrodes resulting in the cleavage of the O<sub>2</sub> bond. After this cleavage, high-energy free radicals can insert themselves into the polymer backbone resulting in the

25 formation of various oxygen moieties, among which are hydroxyl groups. (U.S. Patent No. 5,357,005; U.S. Patent No. 5,132,108)

As used herein, the bifunctional molecule comprises

- (a) a hydroxyl-reactive electrophile, including but not limited to silanes, carboxymethyl groups, succinimides, succinimidyl succinates, benzotriazole carbonates,
- 30 glycidyl ethers (or epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates, aldehydes, isocyanates, and tresylates; and

(b) a nucleophile, including but not limited to sulfhydryl groups, amine groups, hydroxyl groups, or proteins or fragments thereof, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells.

In one embodiment, the bifunctional molecule comprises an organosilane, wherein silane is the electrophile, and the nucleophile includes, but is not limited to sulfhydryl groups, amine groups, hydroxyl groups, or proteins, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells. As used herein, organosilanes fall into a larger class of molecules, which have the capability of forming self-assembled (SA) films. The general form of this molecule comprises  $R_nSiX_{4-n}$ , where  $n = 1, 2,$  or  $3$ ;  $X = Cl, OCH_3,$  or  $OC_2H_5$ , and  $R$  is the nucleophile as described above.

In a preferred embodiment, the bifunctional molecule comprises an aminosilane, wherein silane is the electrophile that attaches to the surface hydroxyl groups, and an amine group is the nucleophilic that can bind to other molecules and/or cells.

In a more preferred embodiment, the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to trimethoxysilylpropyldiethylenetriamine, trimethoxysilylethylenediamine, aminopropyltriethoxysilane, trimethoxyaminopropylsilane, or chlorosilanes such as trichlorosilylethylenediamine, aminopropyltrichlorosilane. In a most preferred embodiment, the amino silane is trimethoxysilylpropyldiethylenetriamine

As used herein, a cell adhesive molecule includes compounds that: (1) introduce charge; and/or (2) are polar; and/or (3) contain sulfur and/or amines; and/or (4) are capable of tethering cells or other cell binding moieties, such as proteins, peptides, and synthetic ligands for cell surface receptors, thereby creating a cell binding location.

As used herein, the term cell repulsive moiety includes compounds that are capable of directly inhibiting cell binding, or that bind to other moieties which inhibit cell binding to the location, including polyethylene glycol (PEG) and other oxygen-rich compounds, sugars, hydrogels, extremely hydrophilic surfaces, or extremely hydrophobic surfaces.

In all of these embodiments, the cell adhesive molecule and/or cell repulsive moiety can be applied to the substrate via solution or vapor phase deposition. In a preferred embodiment, vapor deposition of the cell adhesive molecule and/or cell repulsive moiety is utilized. For example, vapor phase deposition of various silanes has been demonstrated. (Tripp et al., Langmuir 8:1120-1126 (1992); Moses et al., Analytical Chemistry 20:4 (1978))

In most cases, rather than adding the sample to a solution of silane, a hydroxylated surface is placed in the presence of vaporized silane (achievable by traditional vacuum techniques). The reaction takes place at the surface and results in self-assembled monolayers similar to that of silane solution deposition.

5       A wider range of cell adhesive molecules and cell repulsive moieties can be used with vapor phase deposition, because a solvent is not needed. For example, many silane solvents would dissolve the polymeric substrate and destroy its optical quality. In this embodiment, the method circumvents the use of solvents altogether.

10       In another preferred embodiment, the cell repulsive moiety comprises an amine-reactive moiety, including but not limited to 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride)-activated polyethylene glycol (PEG), polyvinylpyrrolidone, polyvinylalcohol, or any other amine-reactive extremely hydrophilic compound such as sugars (mannitol) or PEG, where the amine-reactive part can include, but is not limited to, carboxymethyl groups, succinimides, succinimidyl succinates, benzotriazole carbonates, glycidyl ethers (or  
15       epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates, aldehydes, isocyanates, and tresylates; or any amine-reactive extremely hydrophobic compound such as tridecafluoro-1,1,2,2-tetrahydrooctyl groups (13f) where the amine-reactive part can include, but is not limited to carboxymethyl groups, succinimides, succinimidyl succinates, benzotriazole carbonates, glycidyl ethers (or epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates,  
20       aldehydes, isocyanates, and tresylates. In a most preferred embodiment, the amine-reactive cell repulsive moiety comprises tresyl chloride-activated polyethylene glycol ("tresyl-chloride activated-PEG").

      The chemistry of the tresyl-activated PEG can be used to regulate surface hydroxyl, amine, or sulfhydryl groups. Tresyl chloride will allow stable linkages to be formed between  
25       the nucleophile and the initial hydroxyl, amine, or sulfhydryl group carrying carbon. In a preferred embodiment, PEG is attached to a tresyl group for reaction with surface aminosilane groups.

      In these preferred embodiments, cell adhesive cues can be defined by the use of a stencil, which has no size constraints. Cell repulsive cues, which also can be defined by the  
30       stencil, are tethered to an aminosilane monolayer. The cell binding locations may optionally be coated with cell adhesive proteins, protein fragments, or peptides, and seeded with cells resulting in a patterned array of cells.

This hydroxylated substrate is contacted with a bifunctional molecule comprising an electrophile and a nucleophile. This modified substrate is contacted with a textured elastomeric substrate (herein referred to as a 'stencil'), such as rubber, polyurethanes and poly(dimethyl) siloxanes ("PDMS"), to form a hermetic seal between defined regions of the stencil and the modified substrate. In a preferred embodiment, the stencil comprises PDMS. These materials are quite affordable, providing a significant benefit over traditional UV photolithography methods that employ a costly, high energy laser apparatus. (U.S. Patent No. 5,077,085)

The stencil comprises a physical mask that enables physical protection of defined regions of the underlying substrate from the subsequent solution or vapor phase deposition of the cell repulsive or cell adhesive moiety. This disclosed method of using a 'physical mask' distinguishes itself from existing art that relies on the use of an 'optical mask' (Dulcey et al., Science 252:551 (1991) and U.S. Patent Nos. 5,965,305 and 5,391,463) or 'contact imprinting' (U.S. Patent Nos. 5,512,131 and 5,776,748). The use of optical masks for protecting or de-protecting defined regions of a substrate is limited to the use of photoactivatable chemistries and/or photolabile molecules. The use of 'contact imprinting' is limited to solution phase transfer of materials onto a surface while not enabling 'protection' or 'de-protection' of defined regions of the surface. Further, contact imprinting does not enable reproducible transfer of controlled amounts of material onto the surface. The use of a 'stencil', as disclosed in this invention, allows for protection of a region of the substrate to enable modification of unprotected regions with solution or vapor phase chemistries not limited to photoreactive/photolabile molecules.

The present invention is not constrained to one particular kind of substrate. The tethering chemistry of the primary monolayer, or the organosilane, is such that it reacts with surface hydroxyl groups. These hydroxyl groups can be introduced on the surface of virtually any plastic and glass by low temperature plasma treatment. The secondary tethering chemistry, tresyl chemistry, can react with surface amines, hydroxyl, and sulfhydryls, making it possible to attach to a wider array of surface chemistry. The desired effect is also achievable with high density surface hydroxyl groups, (which may eliminate any silane treatment). (Dust, Macromolecules, 1990. 23:3742-3746; U.S. Patent No. 5,330,911) All of these benefits make the disclosed method of patterning on glass and plastics affordable, facile, and accurate.



The benign nature of the chemistry employed makes it attractive for biological applications, allowing the array to be prepared on glass and any thermoplastic and thermoset of choice including, but not limited to poly(styrene), poly(olefin), poly(dimethyl) siloxane (PDMS), poly(carbonate), poly(vinyl) chloride, poly(ethylene), poly(ethylene) terephthalate, 5 Teflon, and fluorinated ethylene co-poly(propylene) (FEP). The present methods also have the ease and flexibility to be applied to polymeric and glass substrates using the same method. Plastics such as poly(styrene), acrylics, and poly(olefin) have benefits over glass, ceramics and metals because of their affordability, flexibility of shape and size, ease of engineering, durability, low cost and control over its optical quality. The plastics are easily 10 obtained at a minimal cost, can be molded into almost any shape conceivable, and are durable.

The present methods for preparing a substrate for selective cell patterning are more reproducible than are methods that employ contact printing, because there is less opportunity for operator error. There is operator dependence when contact printing due to the subjectivity 15 of applying the stamp to the substrate (force by which the stamp is depressed, amount of solution on the stamp) and so the results will vary. (U.S. Patent No. 5,776,748) The present method of using a stencil for masking while performing solution or vapor phase deposition of the cell adhesive molecules and/or cell repulsive moieties is operator independent, thus providing a scalable and manufacturable process.

20 The instantly disclosed method of cell patterning has a marked advantage over prior thiol chemistry. Previous technology of contact printing with thiols not only introduces operator error, but also requires a thin layer of gold to be evaporated on the surface of the tissue culture substrate. Due to the high temperature involved with gold evaporation, most plastics cannot be used. Optical quality is constrained and fluorescence light is absorbed due 25 to the added layer of gold, which reduces the quality of information gathered when conducting cell-based screening. In addition to a lower optical quality, there is a high cost associated with gold coating. Furthermore, silane linkages are covalent, and are not subject to degradation, as are thiols on gold, which degrade over time due to impurities and the fact that a thiol bond is coordinate and not covalent. The methods of the present invention permit cell 30 patterning on an optically clear substrate and give the added option of control over the substrate, so that one has the freedom to choose the most superior affordable plastic or glass for optical quality.

In a particular embodiment of the

present method, oxygen plasma is used to activate the surface in the case of poly(styrene) and poly(olefin), and acid washing is used to activate the surface in the case of glass. Both surfaces can be further incubated with a mildly acidic alcoholic solution of aminosilane featuring a primary amine on the terminating end of the tethered molecule. Following silane treatment, a stencil is applied to the substrate. An aqueous solution of tresyl chloride-activated PEG is applied to the substrate around the stencil resulting in regions of exposed amine, and regions of PEG in carefully controlled proximity to one another. After surface modification, the surface can be primed with cell adhesive proteins, protein fragments, or peptides to speed the cell adhesion process. (U.S. Patent No. 5,874,219)

In another aspect, the present invention provides novel patterned substrates for cell culture. In one aspect, the invention provides cell patterning substrates, comprising:

1. at least a first portion having a reactive surface to which a plurality of cell adhesive molecules are coupled;
2. and at least a second portion having an exposed surface to which a plurality of cell repulsive moieties are coupled; wherein the cell adhesive molecules are selected from the group consisting of silanes, and wherein the cell repulsive moieties comprise tresyl-chloride activated poly(ethylene) glycol.

In a preferred embodiment, the silane comprises  $R_nSiX_{4-n}$ , where  $n = 1, 2$ , or  $3$ ;  $X = Cl$ ,  $OCH_3$ , or  $OC_2H_5$ ;  $R =$  a nucleophile, including but not limited to sulfhydryl groups, amine groups, hydroxyl groups, charged groups, polar groups, or proteins, protein fragments, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells. In a preferred embodiment, the silane is an aminosilane. In a more preferred embodiment, the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to trimethoxysilylpropyldiethylenetriamine, trimethoxysilylethylenediamine, aminopropyltriethoxysilane, trimethoxyaminopropyl-silane, or chlorosilanes such as trichlorosilylethylenediamine, aminopropyltrichlorosilane. In a most preferred embodiment, trimethoxysilylpropyldiethylenetriamine is used.

In another embodiment, the substrate further comprises cell adhesive proteins, protein fragments, or peptides, including but not limited to fibronectin, laminin, collagen, vitronectin, osteopontin, RGD peptides, RGDS peptides, YIGSR peptides. The strength of cell adhesion to the cell adhesion promoters can be modified by varying the composition of the cell adhesive proteins, protein fragments, or peptides. In a further embodiment, the substrate

further comprises cells bound to the cell binding locations, either directly or indirectly via cell adhesive proteins, protein fragments, or peptides. Any cell type may be used, including prokaryotic, eukaryotic, and archaeobacterial cells.

5 The cell binding locations according to the various methods and substrates of the invention can be as small as the diameter of a single cell and as large as several hundred cell diameters. The distance between cell binding locations (i.e.: the cell repulsive locations) is cell size dependent, but is sufficiently large so that a cell cannot bridge the gap between cell binding locations (i.e.: 1 cell diameter), unless a particular application calls for interaction of cells in different cell binding locations.

10 In a further embodiment, the various cell patterning substrates are mated with a fluid delivery system to provide fluid and/or reagent flow to the cell binding location. In a preferred embodiment, the fluid delivery system is that described herein.

In another embodiment, the cell patterning substrate comprises a cell patterning substrate made by the methods of the invention, as disclosed above.

15 This aspect of the present invention may be better understood with reference to the accompanying preferred embodiments that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

## 20 **Materials and Methods:**

Reagents and instrumentation that can be utilized in carrying out the methods of the invention include, but are not limited to, 60 and 35 mm petri dishes, microplates, thermoplastics, poly(olefin), plasma cleaner/sterilizer, digital convection gauges, trimethoxysilylpropyldiethylenetriamine, and 2,2,2-trifluoroethanesylphenyl-  
25 poly(ethylene)<sub>5000</sub> glycol.

Poly(styrene), poly(olefin), or other thermoplastic substrates such as poly(esters) and poly(ether) are oxygen plasma treated inside a plasma cleaner using the following method. Substrates are placed inside the glass tube chamber and the chamber is evacuated to a pressure of ~200 mtorr as indicated by a convection gauge. Oxygen is pulsed in through a  
30 regulation valve and the chamber is evacuated again to a pressure of ~200 mtorr. The above oxygen pulse is repeated 2 more times. After the last oxygen pulse, the gas is allowed to bleed constantly into the chamber, and the final equilibrium pressure (with the oxygen bleed

We claim

- 5 1. A method of making a substrate for selective cell patterning comprising:
- a) providing a substrate with a surface, wherein the surface contains reactive hydroxyl groups;
  - b) contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to  
10 form a monolayer;
  - c) applying a stencil to the substrate;
  - d) applying an electrophilic cell repulsive moiety to exposed regions of the monolayer to form a covalent bond between the cell repulsive moiety and the bifunctional nucleophile deposited in step b, to produce cell repulsive locations;
  - 15 e) removing the stencil; and
  - f) applying cell adhesive molecules to the substrate to produce cell binding locations, wherein the cell adhesion molecules bind the substrate only in positions that were contacted by the stencil.
- 20 2. A method of making a substrate for selective cell patterning comprising:
- a) providing a substrate with a surface, wherein the surface comprises reactive hydroxyl groups on the surface of the substrate;
  - b) contacting the hydroxyl groups on the surface of the substrate with a bifunctional molecule comprising a hydroxy-reactive moiety and a nucleophilic moiety to  
25 form a monolayer
  - c) applying a stencil to the substrate;
  - d) applying cell adhesive molecules to exposed regions on the monolayer to produce cell binding locations;
  - e) removing the stencil; and
  - 30 f) applying an electrophilic cell repulsive moiety to the substrate to produce cell repulsive locations, wherein the cell repulsive moiety forms a covalent bond with the

bifunctional nucleophile deposited in step b only in positions that were contacted by the stencil.

3. The method of claim 1 or 2 wherein the substrate is treated to provide the reactive  
5 hydroxyl groups on the surface of the substrate.

4. The method of claim 3 wherein the substrate is oxygen plasma treated to provide the reactive hydroxyl groups on the surface of the substrate.

10 5. The method of claim 1-2 wherein the bifunctional molecule comprises an organosilane

6. The method of claim 1-2 wherein the bifunctional molecule comprises an aminosilane.

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7. The method of claim 1-2 wherein the reactive cell repulsive moiety comprises a tresyl-chloride activated-poly(ethylene) glycol compound.

8. The method of claim 1-2 wherein vapor phase deposition is used for the contacting of  
20 the hydroxyl groups on the surface of the substrate with a bifunctional molecule.

9. The method of claim 1-2 further comprising contacting the cell binding locations with a cell-binding protein or peptide under conditions to permit binding of the cell-binding protein or peptide to the cell binding location.

25

10. The method of claim 1-2 further comprising contacting the cell binding locations with cells under conditions to permit binding of the cells to the cell binding locations.

11. The method of claim 9 further comprising contacting the cell-binding protein or  
30 peptide on the cell binding locations with cells under conditions to permit binding of the cells to the cell-binding protein or peptide.

12. The method of claim 9 further comprising lyophilizing the substrate.
13. The method of claim 10 further comprising cryopreserving or desiccating the substrate.
- 5 14. The method of claim 11 further comprising cryopreserving or desiccating the substrate.
- 10 15. A cell patterning substrate, comprising at least a first portion having a reactive surface to which a first plurality of cell adhesive molecules are coupled, and at least a second portion having an exposed surface to which a second plurality of cell repulsive moieties are coupled, wherein the cell adhesive molecules are selected from the group consisting of silanes, and wherein the cell repulsive moieties comprise tresyl chloride-activated poly(ethylene) glycol.
- 15 16. The cell patterning substrate of claim 15, wherein the silane comprises an aminosilane.
17. The cell patterning substrate of claim 16, wherein the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to  
20 trimethoxysilylpropyldiethylenetriamine, trimethoxysilylethylenediamine, aminopropyltriethoxysilane, trimethoxyaminopropyl-silane, or chlorosilanes such as trichlorosilylethylenediamine, aminopropyltrichlorosilane.
18. The cell patterning substrate of claim 16, wherein the aminosilane is  
25 trimethoxysilylpropyldiethylenetriamine.
19. The cell patterning substrate of claim 15, further comprising cell-binding proteins or peptides bound to the plurality of cell adhesive molecules.
- 30 20. The cell patterning substrate of claim 19, wherein the substrate is lyophilized.

21. The cell patterning substrate of claim 15, further comprising cells bound to the plurality of cell adhesion promoters.
22. The cell patterning substrate of claim 19, further comprising cells bound to the cell-binding proteins or peptides.
23. The cell patterning substrate of claim 21, wherein the substrate is cryopreserved or subjected to desiccation.
24. The cell patterning substrate of claim 22, wherein the substrate is cryopreserved or subjected to desiccation.
25. A cell patterning substrate, prepared by the method of claims 1-2.

Figure 2

